Polyhydroxylated C60 fullerene (fullerenol) attenuates neutrophilic lung inflammation in mice

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Abstract:

Inflammation is crucial to eliminate pathogens and promote repair of injured tissue. However, excessive or persistent inflammation can contribute to tissue injury and the pathogenesis and exacerbation of diseases, including inflammatory lung diseases, such as chronic obstructive pulmonary disease [**1**] and silicosis [**2**]. Neutrophilic inflammation is an important aspect of chronic obstructive pulmonary disease [**1**, **3**, **4**] and silicosis [**2**, **5**]. Thus, in human beings, a relationship between exposure to respirable silica in coal mine dust and pulmonary inflammation is seen, resulting in an elevated neutrophil count in bronchoalveolar lavage fluid (BALF) [**6**]. Exposure to silica can cause silicosis, where the severe inflammation in the lung appears to be an initiating step in the development of the disease [**7**]. The quartz particles can in itself generate reactive oxygen species (ROS), but additional inflammatory injuries appear to be a result of the influx of inflammatory cells [**2**]. The cell-generated ROS and nitric oxide radicals are hallmarks of the toxicity of the quartz particles [**8**, **9**], and quartz-induced inflammation is characterised by, for example, neutrophilic inflammation in rodents [**10**]. The importance of neutrophils in the development of inflammatory lung diseases has been reported in rodents, where exposure to quartz resulted in induced influx of neutrophils [**2**, **4**, **11**-**15**]. Furthermore, it was shown that treatment with anti-macrophage inflammatory protein 2 (MIP-2) antiserum prior to quartz exposure attenuated neutrophil influx [**16**], suggesting that MIP-2 can play an important role in quartz-induced neutrophilic lung inflammation.

Keywords: inflammatory lung disease | pulmonary inflammation | neutrophils | fullerenol

Article:

Inflammation is crucial to eliminate pathogens and promote repair of injured tissue. However, excessive or persistent inflammation can contribute to tissue injury and the pathogenesis and exacerbation of diseases, including inflammatory lung diseases, such as chronic obstructive pulmonary disease [**1**] and silicosis [**2**]. Neutrophilic inflammation is an important aspect of chronic obstructive pulmonary disease [**1**, **3**, **4**] and silicosis [**2**, **5**]. Thus, in human beings, a relationship between exposure to respirable silica in coal mine dust and pulmonary inflammation is seen, resulting in an elevated neutrophil count in bronchoalveolar lavage fluid (BALF) [**6**]. Exposure to silica can cause silicosis, where the severe inflammation in the lung appears to be an initiating step in the development of the disease [**7**]. The quartz particles can in itself generate reactive oxygen species (ROS), but additional inflammatory injuries appear to be a result of the influx of inflammatory cells [**2**]. The cell-generated ROS and nitric oxide radicals are hallmarks of the toxicity of the quartz particles [**8**, **9**], and quartz-induced inflammation is characterised by, for example, neutrophilic inflammation in rodents [**10**]. The importance of neutrophils in the development of inflammatory lung diseases has been reported in rodents, where exposure to quartz resulted in induced influx of neutrophils [**2**, **4**, **11**-**15**]. Furthermore, it was shown that treatment with anti-macrophage inflammatory protein 2 (MIP-2) antiserum prior to quartz exposure attenuated neutrophil influx [**16**], suggesting that MIP-2 can play an important role in quartz-induced neutrophilic lung inflammation.

Development of novel anti-inflammatory drugs is an important issue. One mechanism by which inflammation can be attenuated is by elimination of ROS and free radicals [**17**, **18**]. Fullerenes, a recently discovered allotrope of carbon [**19**], have attracted much attention in pharmacology as reviewed [**20**]. Fullerenes, also termed buckminsterfullerenes or simply 'bucky balls'[**21**], are molecules consisting of 60 or more carbon atoms arranged in a soccer ball-like structure. The pristine C_{60} fullerene has 30 conjugated C–C double bonds and may therefore be chemically derivatized which may modify the physicochemical and pharmacological properties of the molecule. The water-soluble hydroxylated bucky balls is well known to possess ROS and radical scavenging effects [**20**], that is, they may be able to neutralize ROS and thereby probably also attenuate inflammation induced by ROS [**22**, **23**].

Using mice as the model animal, this study examines whether pre-treatment with polyhydroxy C_{60} (fullerenol) can attenuate neutrophilic lung inflammation induced by quartz.

Materials and methods

Fullerenol, $C_{60}(OH)_{20\pm2}$, was purchased from BuckyUSA (Houston, TX, USA). Quartz (crystalline α-quartz SRM 1878a, median size 1.6 μm) was purchased from the National Institute of Standards and Technology (Washington, DC, USA). Fullerenol and quartz were dissolved and suspended, respectively, in pyrogen-free saline and administered to 7–8 weeks old (19.6–21.9 g) BALB/cJ female mice (Taconic, Ry, Denmark) by intratracheal instillation. The mice were orotracheally intubated with a flexible polyethylene catheter under deep Hypnorm®/Dormicum® (VetaPharma, Leeds, UK/Roche, Basel, Switzerland) anaesthesia. Twenty-four hours after the administration of particles, mice were euthanized under Hypnorm®/Dormicum® anaesthesia, and BALF was collected as previously described [**24**].

Levels of MIP-2 in the BALF were measured by ELISA (R&D systems, Minneapolis, MN, USA), according to the manufacturer's instructions.

After collection of BALF, lungs were filled intratracheally with fixative (10% buffered formalin solution) using a ligature around the trachea. The lungs were removed, placed in the same fixative for further fixation and processed into paraffin. Sections of 5 µm were stained with haematoxylin/periodic acid Schiff, and the presence of inflammatory changes was judged microscopically.

Initially, a study was carried out to study the inflammatory potential of the fullerenol *per se*. Fullerenol was administered in doses of 0.02, 0.2, 2, 20 and 200 µg/mouse dissolved in 40 µl pyrogen-free isotonic saline given as an intratracheal bolus. Furthermore, to study interactions between quartz and fullerenol, 0.2, 2 and 20 µg fullerenol, respectively, was administered in 20 µl saline as an intratracheal bolus 2 min. prior to intratracheal instillation of 50 µg quartz suspended in 20 µl saline, that is, quartz and fullerenol were not mixed *in vitro* before instillation.

Exposure groups were compared pair-wise to the control group by the Mann–Whitney *U* test. Statistically significance was assumed with a P-value less than 0.05.

Discussion

Mice were exposed intratracheally to 0.02, 0.2, 2, 20 and 200 µg fullerenol. BAL data indicated a dose-dependent lung inflammation primarily caused by neutrophils (**fig. 1**). Thus, the 200 µg level of fullerenol induced highly increased neutrophil influx in the lungs, while 20 µg fullerenol was the highest dose tested that did not give rise to lung inflammation. The increase in neutrophils was associated with an increased level of MIP-2, which is a neutrophil attractant (**table 1**).

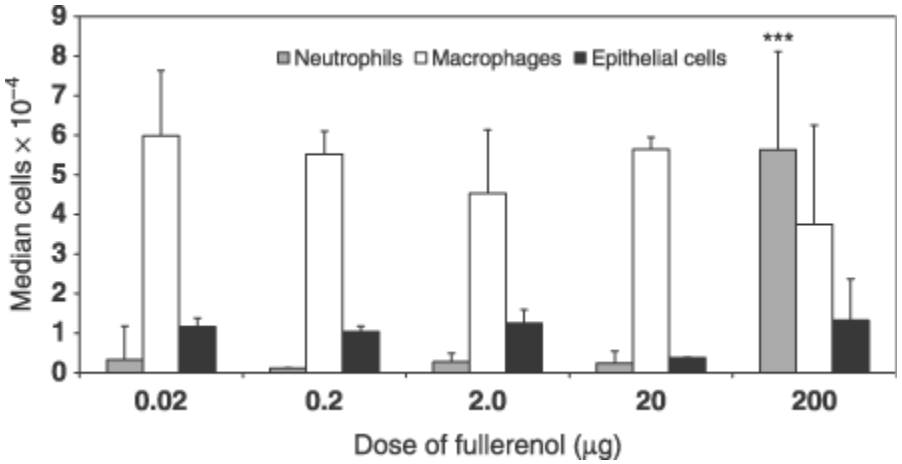

Figure 1. Mice were instilled intratracheally with various doses of fullerenol. Twenty-four hours after instillation, bronchoalveolar lavage was performed and cells were counted. Bars represent the median value of seven to eight mice together with the 75th percentile. A P-value < 0.001 is indicated by asterisks (***).

Histological examination showed no major inflammation 24 hr after instillation of 50 µg quartz (data not shown). Sayes and co-workers reported that intratracheal instillation of $C_{60}(OH)_{24}$ in

rats showed no increased neutrophil number at a low-dose level (0.2 mg/kg), whereas marked inflammation was seen 24 hr after administration of 1.5 mg/kg [**11**]. This is in accordance with our observations in mice where no inflammation was seen at doses up to 20 μg fullerenol/mouse (≈1 mg/kg) but inflammation was seen at 200 μg/mouse (≈10 mg/kg). Assessed on the number of neutrophils, quartz is more potent than fullerenol to induce inflammation as 50 μg quartz gave rise to a more pronounced neutrophil response than 200 μg fullerenol (**figs 1 and 2**).

Table 1. Levels of macrophage inflammatory protein 2 (MIP-2) in bronchoalveolar lavage fluid (BALF).

Treatment	Number of mice in group	MIP-2 (pg/ml BALF)
Saline	8	0.2 ± 0.2
0.02 μg fullerenol	7	0 ± 0
0.2 μg fullerenol	8	0 ± 0
2 μg fullerenol	8	1.4 ± 1.4
20 μg fullerenol	8	0.2 ± 0.2
200 μg fullerenol	8	9.4 ± 0.9[1]
Saline + 50 μg quartz	23	11.4 ± 1.2
0.2 μg fullerenol + 50 μg quartz	8	13.3 ± 2.1
2 μg fullerenol + 50 μg quartz	8	5.6 ± 1.6[2]
20 μg fullerenol + 50 μg quartz	8	10.8 ± 2.8

MIP-2 levels are expressed as mean ± S.E.M. Statistical significant difference when compared to saline control or quartz control is indicated by superscript numbers ([1]and [2]), respectively (Mann–Whitney *U* test).

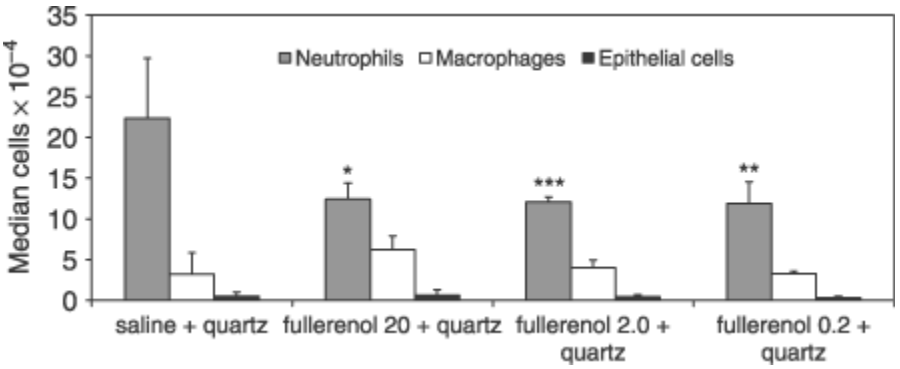

Figure 2. Mice were instilled intratracheally with either saline (control) or various doses (in μg) of fullerenol followed by intratracheal instillation of a fixed dose (50 μg) of quartz. Twenty-four hours after instillation bronchoalveolar lavage was performed and cells were counted. Bars represent the median value of the group together with the 75th percentile. Group sizes are given in **table 1**. P-values less than 0.05, 0.01 and 0.001 are indicated by asterisks (*, ** and ***, respectively).

As seen from **fig. 2**, instillation of 50 μg quartz gave rise to a marked increase in the number of neutrophils in BAL. When mice were pre-treated with fullerenol, the quartz-induced neutrophilic lung inflammation was attenuated. A statistically significantly reduced neutrophil number when compared pair-wise to the quartz control group was seen both at the 20 μg (P = 0.02), 2 μg (P = 0.0002) and 0.2 μg (P = 0.004) fullerenol dose levels, respectively. Pre-treatment with fullerenol was able to reduce the neutrophilic response by approximately 50%. The anti-inflammatory effect at the 2 μg fullerenol level was also associated with a reduced MIP-2 level when compared to the quartz control group (**table 1**). However, no dose–response relationship was seen on the anti-inflammatory effect of the fullerenol. It is tempting to speculate that instillation of a fixed dose of quartz, in this case 50 μg, may lead to formation of a certain amount of ROS. If this is the case, it could be suggested that 0.2 μg fullerenol is sufficient to

neutralize the amount of ROS formed, and hence administration of higher doses of fullerenol will not further attenuate the ROS-induced inflammation. As the quartz-induced inflammation may also be driven by factors other than ROS formation [**13**], the fullerenol cannot be expected entirely to inhibit the inflammation.

By the consecutive intratracheal instillations, there is a possibility that the administered doses of quartz and fullerene do not enter the same parts of the airways. If so, our results would underestimate rather than overestimate the anti-inflammatory effect of fullerenol. A more comprehensive study of an anti-inflammatory effect of water-soluble fullerenes should include aerosol administration, which ensures a better distribution of substance in the airways.

Nevertheless, our data show that fullerenol has an anti-inflammatory effect at lower doses but a pro-inflammatory effect at higher doses (**figs 1 and 2**). A similar phenomenon has been reported for other antioxidants, including vitamin E and carotenoids [**25**].

In conclusion, the present study shows that quartz-induced neutrophilic inflammation in the lungs can be attenuated by administration of the water-soluble polyhydroxy fullerene derivative, which may be therapeutically relevant as several difficult-to-treat lung diseases are associated with neutrophilic inflammation.

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